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MECHANISMS OF RESISTANCE

Antibiotic Resistance Plasmids Cointegrated into a Megaplasmid Harboring the $bla_{OXA-427}$ Carbapenemase Gene

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ABSTRACT OXA-427 is a new class D carbapenemase encountered in different species of *Enterobacteriaceae* in a Belgian hospital. To study the dispersal of this gene, we performed a comparative analysis of two plasmids containing the $bla_{OXA-427}$ gene, isolated from a *Klebsiella pneumoniae* strain and an *Enterobacter cloacae* complex strain. The two IncA/C2 plasmids containing $bla_{OXA-427}$ share the same backbone; in the *K. pneumoniae* strain, however, this plasmid is cointegrated into an IncFIb plasmid, forming a 321-kb megaplasmid with multiple multiresistance regions.

KEYWORDS carbapenemase, megaplasmids, OXA-427

The dissemination of carbapenem resistance in *Enterobacteriaceae* is a major public health concern, as carbapenem antibiotics are a last-resort therapy for these common pathogens. The new OXA-427 carbapenemase presents a profile of wide resistance to β-lactams, including broad-spectrum penicillins, extended-spectrum cephalosporins, and carbapenems (1, 2). The $bla_{OXA-427}$ gene was detected in different *Enterobacteriaceae* species ($n = 6$) and in different patients ($n = 9$) hospitalized at a university hospital in Belgium, in different hospital wards, between March 2012 and June 2014 (1); this suggests a high level of interspecies transmission of this resistance gene, probably associated with horizontal gene transfer of a specific plasmid. Recent studies, based on long-read sequencing data, suggest that mobility of resistance genes occurs at multiple nested genetic levels, with transmission of strains between individuals, transfer of plasmids between strains, and transposition of resistance genes between plasmids (3, 4). We report the comparative analysis of two plasmids containing the $bla_{OXA-427}$ gene, for future study of the potential mechanism of interspecies transmission of the $bla_{OXA-427}$ gene.

Two OXA-427-producing isolates, one of *Klebsiella pneumoniae* (KLPN57) and the other of the *Enterobacter cloacae* complex (ENCL58), were cultured from clinical samples from two different patients, in January 2013 and October 2013, respectively. Genomic DNA was extracted from these isolates and PacBio sequencing was performed at the Genomics Core laboratory (Leuven, Belgium), according to the protocol used by Conlan et al. (5). Genomic DNA was sheared into 10- to 15-kb fragments and converted into SMRTbell template libraries. Each strain was sequenced on a single SMRT cell with the PacBio RSII system, using P6 polymerase binding and C4 sequencing kits. The sequencing coverage for both plasmids ranged from 160-fold to 400-fold coverage. Genome assemblies were performed using HGAP 3 as part of SMRTAnalysis 2.3.0, annotation of the genes was performed by using RAST (http://rast.nmpdr.org), and the

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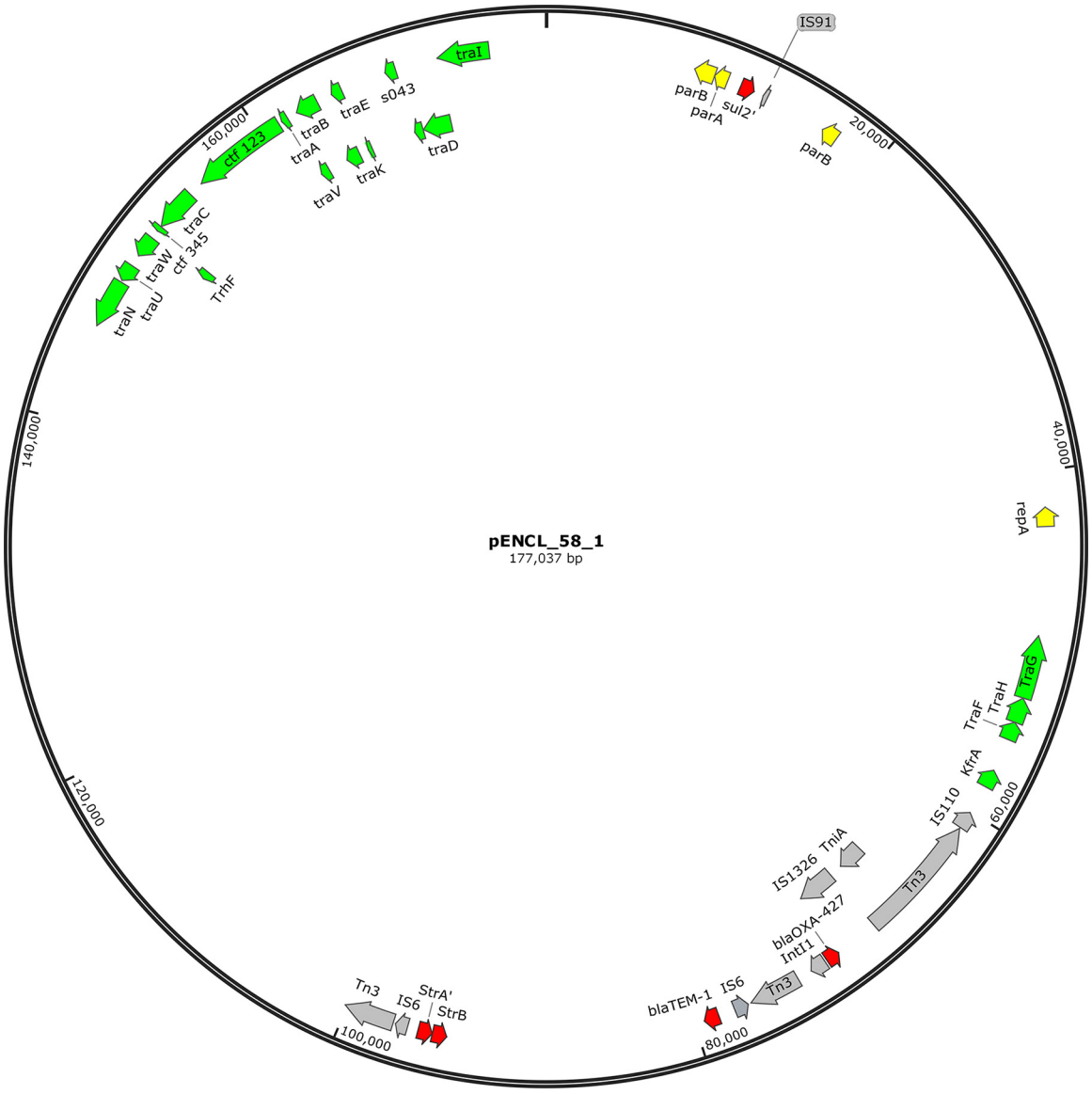

FIG 1 Circular representation of pENCL58_01, harboring $bla_{OXA-427}$. Only genes for partitioning/replication (yellow), conjugative transfer (green), antibiotic resistance (red), and mobility (gray) are depicted. Prime symbols indicate truncated genes.

functional assignments for the predicted open reading frames (ORFs) were confirmed by using BLASTP with the Protein Data Bank (PDB) from NCBI (http://www.ncbi.nlm.nih.gov). IS finder and ResFinder were used for identification of mobile elements and antibiotic resistance genes, respectively (6, 7). PubMLST was used for multilocus sequence typing (MLST) (8). The plasmid maps were drawn by using SnapGene software.

The genomic content of *Enterobacter cloacae* strain ENCL58 consists of a 5.0-Mb genome with sequence type 171 (ST-171) and three plasmids (177 kb, 121 kb, and 84 kb). The 177,037-bp plasmid pENCL58_01, carrying the $bla_{OXA-427}$ gene, is an IncA/C2 plasmid of plasmid multilocus sequence type 3, 100% identical to KX869741.1 (1) (Fig. 1). The other two plasmids (IncFIb and IncFII types) carry no known resistance genes. The IncA/C2 plasmid shows overall high levels of homology with other IncA/C2 plasmids found in *Enterobacter aerogenes* (GenBank accession number FO203354.1; 99% sequence identity and 87% coverage), *Klebsiella pneumoniae* (GenBank accession number JQ010984.1; 99% sequence identity and 86% coverage), and *Salmonella en-*

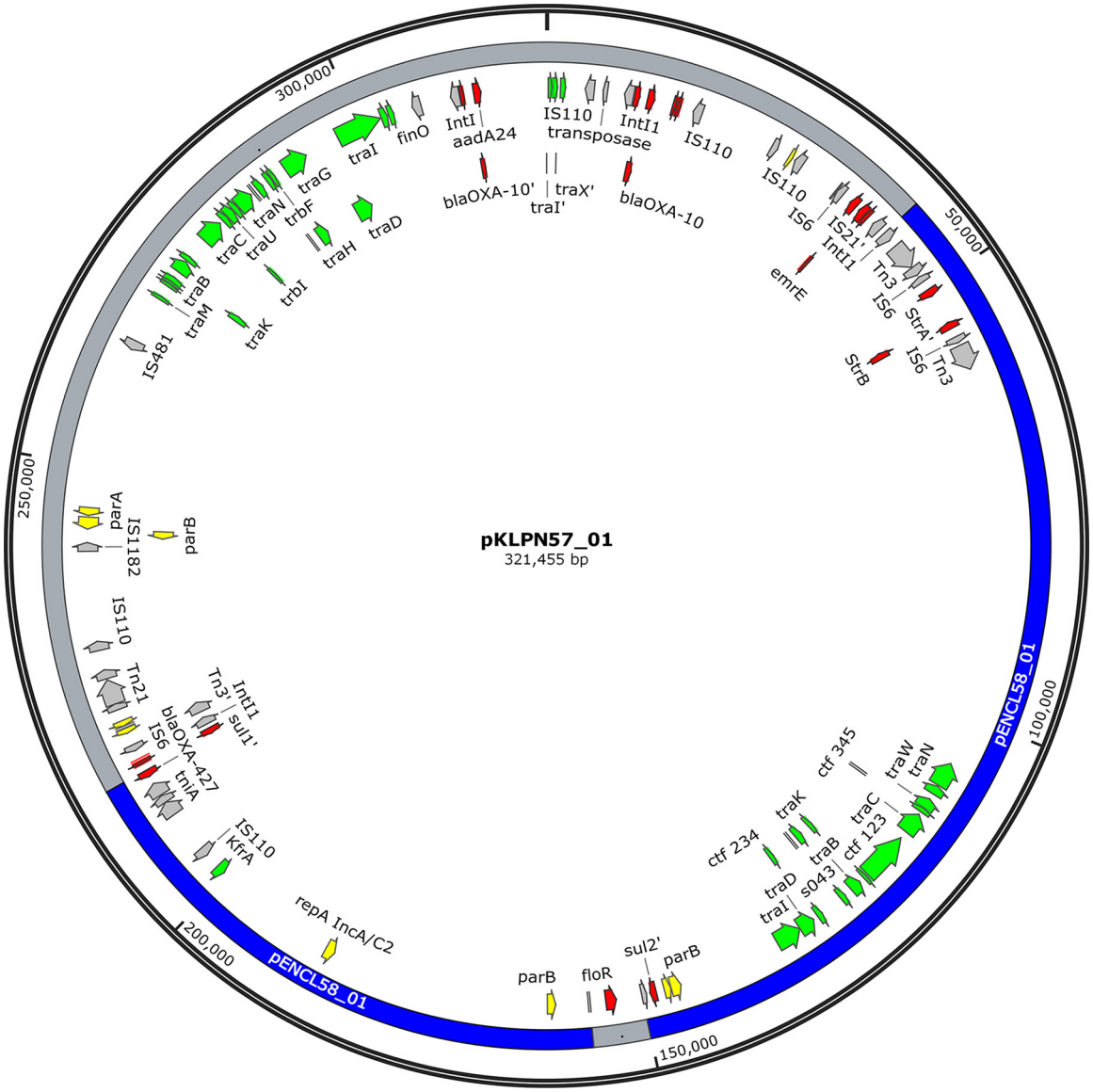

FIG 2 Circular representation of pKLPN57_01, harboring $bla_{0XA-427}$. The plasmid parts identical to those in pENCL58_01 (>99% similarity) are indicated in blue. Only genes for partitioning/replication (yellow), conjugative transfer (green), antibiotic resistance (red), and mobility (gray) are depicted. Prime symbols indicate truncated genes.

terica (GenBank accession number KM670336.1; 99% sequence identity and 75% coverage). The plasmid pENCL58_01 contains 223 coding sequences, including three functional antibiotic resistance genes ($bla_{OXA-427}$, bla_{TEM-1B}, and *strB*) and two truncated resistance genes (*strA* and *sul2*). The bla_{TEM-1} gene and the *strB-strA* gene combination are located adjacent to the $bla_{OXA-427}$ resistance transposon, in the direction away from *repA*, and both are associated with the presence of IS*15* elements, making this a resistance island (RI). In this plasmid, two transfer regions were identified, one located from position 49313 to position 57324 and including 3 *tra* genes and *kfrA* (for plasmid maintenance) and one located from position 145665 to position 173831 and including 12 *tra* genes and *trhF*.

The assembly of the *Klebsiella pneumoniae* strain KLPN57 reads resulted in a genome of 5.23 Mb with ST-1 and three plasmids (321 kb, 175 kb, and 77 kb). The $bla_{OXA-427}$ gene was identified on the 321,455-bp plasmid pKLPN57_01, which carries 417 predicted coding sequences (Fig. 2). The other plasmids are of type IncFIb, with resistance genes $bla_{CTX-M-15}$, bla_{TEM-1B}, *aph(3′)-Ia*, *aadA2*, and *sul1*, and type IncFII, with *qnrS1*.

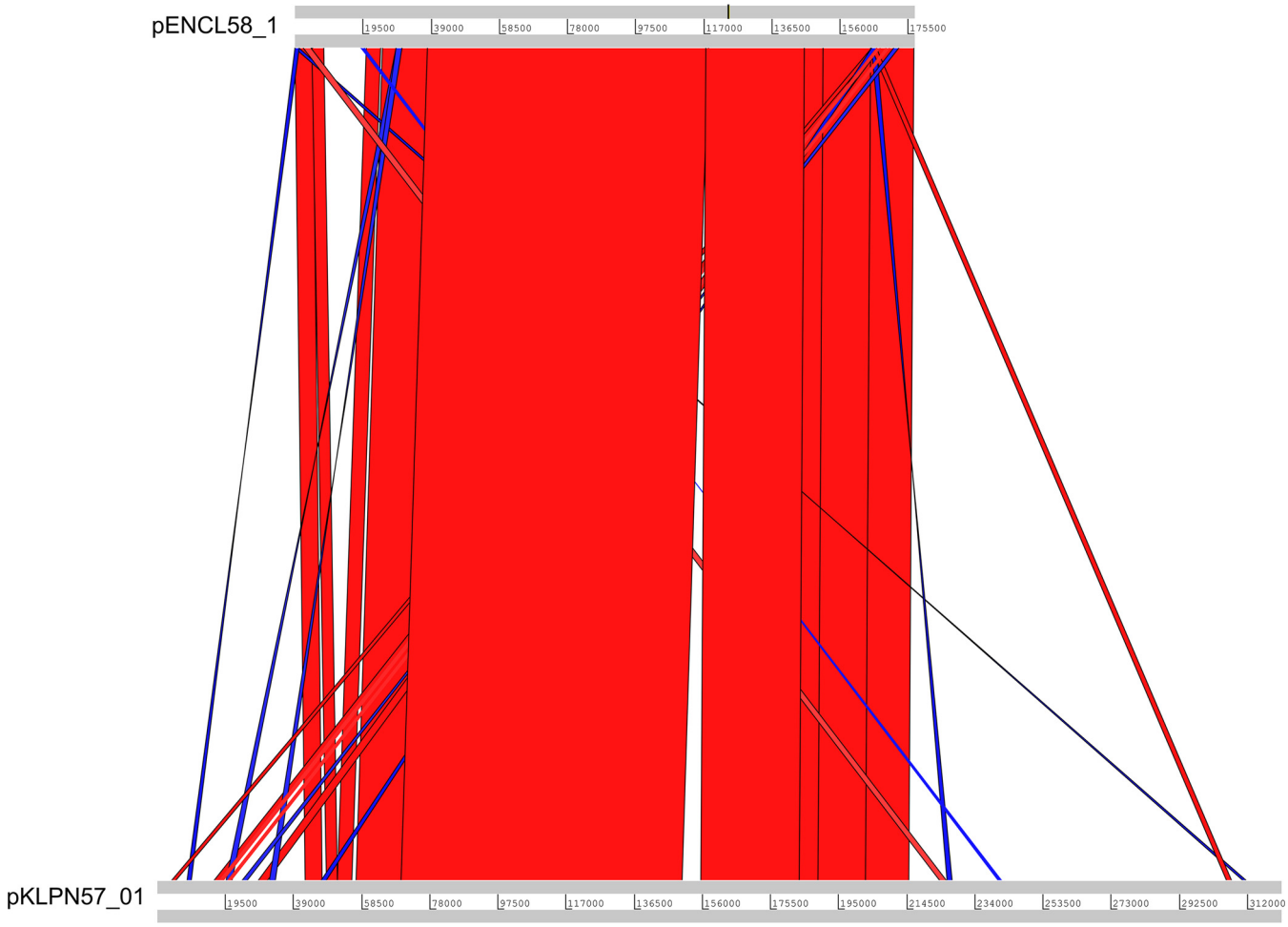

FIG 3 Pairwise comparison of pENCL58_01 (upper sequence) and pKLPN57_01 (lower sequence). The *intI1* gene of pENCL58_01 is used as a starting point. At the starting position, the homologous plasmid is disrupted and integrated into the pKLPN57_01 megaplasmid. Connecting bars represent regions with BLASTN matches of >99.9% in the same (red) and inverted (blue) orientations, visualized with the Artemis Comparison Tool (13).

The plasmid pKLPN57_01 contains resistance genes for aminoglycoside resistance (*aadA24*, *aacA4*, and *strB*), β-lactam resistance (bla_{OXA-10}, bla_{TEM-1B}, and $bla_{OXA-427}$), chloramphenicol resistance (*floR*), and sulfonamide resistance (*sul1*). Five additional resistance genes (*strA*, bla_{OXA-2}, *sul2*, bla_{OXA-10}, and *aacA4*) are truncated and potentially dysfunctional. Interestingly, two types of *repA* genes, coding for plasmid replication proteins, were detected on that plasmid. These *repA* genes belong to two distinct plasmid incompatibility groups, namely, IncA/C2 and IncFIb. Further analysis confirmed that pKLPN57_01 is composed of a novel plasmid belonging to the IncFIb group and an IncA/C2 plasmid highly similar to pENCL58_01, which was identified in *Enterobacter cloacae* ENCL58 (Fig. 3). Additionally, the plasmid carries two parts of a *repA* gene related to *colW* separated by the IncA/C2 region, together constituting the complete replication gene. Based on the presence of different *repA* genes and the large number of mobile genetic elements, the pKLPN57_01 plasmid is in fact a megaplasmid of 321 kb and could be a result of multiple fusions of different plasmids and/or mobile elements. The IncFIb region showed the highest level of homology with a *K. pneumoniae* plasmid (GenBank accession number CP021541.1; 99% identity and 68% coverage) and the *colW* region with a *Achromobacter xylosoxidans* subsp. *denitrificans* plasmid (GenBank accession number HF679279.1; 100% identity and 37% coverage). Unfortunately, we were not able to identify the sources of these plasmid elements.

The genetic environment downstream of the $bla_{OXA-427}$ gene, characterized by

IS*1326* and a Tn*3* family transposon, is identical in the two plasmids. Upstream of the $bla_{OXA-427}$ gene, however, additional resistance genes are present in pKLPN57_01, compared to pENCL58_01 (Fig. 1 and 2).

In Fig. 3, the alignment of pENCL58 and pKLPN57 is shown. In the pKLPN57_01 megaplasmid, the IncA/C2 region is flanked by identical class I integron integrase genes, *intI1*, at both sites. At the 5′ site, the *intI1* gene is fused with a resistance integron. At the 3′ site, the *intI1* gene of IncA/C2 is fused with a partial *colW*-like *repA* gene by a transposase gene of the Tn*3* family and an IS*6* insertion sequence. In both regions of fusion, there is an abundance of Tn*3* family transposases and IS*6* and IS*110* insertion sequences. The region homologous to pENCL58 is divided into two parts by a transposon flanked on both sides by IS*91*, carrying the resistance gene *floR*.

We conclude that the megaplasmid in the *K. pneumoniae* isolate is a result of cointegration of an IncA/C2-type plasmid harboring $bla_{OXA-427}$ with an IncF1b-type plasmid. The ability of Tn*3* and insertion sequences to mediate cointegration of plasmids is well known (9). Plasmid cointegrates have been reported previously for carbapenemase-producing *Enterobacteriaceae* strains (5, 10); they showed IncFIa-type plasmid backgrounds and IS*26* insertion sequences flanking the regions of fusion. To our knowledge, however, cointegrates formed from large resistance plasmids with sizes of >300 kb have not been reported previously. The plasmid cointegration seems to represent an important molecular pathway for interspecies transfer of plasmids and their resistance genes. Cointegration with disruption of replicons, as observed in pKLPN57_01, may allow plasmid incompatibility to be overcome (11). Next, cointegration with a plasmid that has coevolved in *K. pneumoniae* may provide the transferred plasmid with specific conjugation and restriction modification machinery (12). The cointegration of plasmids into pKLPN57_01 has probably taken place in recent years. Future studies will need to clarify whether megaplasmids of the size presented in our study are persistent or represent a temporary initial stage in the evolution of antibiotic resistance plasmids.

Accession number(s). The annotated plasmid sequences of pKLPN57_01 and pENCL58_01 were deposited in DDBJ/EMBL/GenBank under accession numbers LT882698 and LT882699, respectively.

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